Foxg1 null allele mice exhibit frontal bone developmental abnormalities

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Tiivistelmä - Referat – Abstract

Forkhead box G1 proteiini (FOXG1) on transkriptiorepressori, joka osallistuu moniin eri signaalireitteihin. Se on aiemmalta nimeltään Brain Factor 1 (BF1) ja on tunnettu vaikutuksistaan neurogeneesiin. FOXG1 expressoituu esiaivoissa ja kasvojen alueen hermostopienan soluissa.

Tutkimuksen tavoitteena oli tutkia FOXG1:n osuutta otsaluiden kehityksessä hiirillä. Tämä toteutettiin Foxg1-mutatoidun hiirilinjalla avulla, jossa Foxg1-alleeli korvattiin Cre-rekombinaasilla. Tutkimuksessa käytettiin geenitekniikan menetelmiä ja luuston analyysejä. Ero mutantti alkioiden $Foxg1^{cre/cre}$ ja villityypin alkioiden $Foxg1^{+/+}$ välillä löytyi.

$Foxg1^{cre/cre}$ mutanttialkioiden otsaluiden ja nenäluiden alueelta löytyi poikkeavuuksia. Otsaluiden välinen sauma oli tähden mallinen ja otsaluut olivat kapeampia, sekä kallot olivat pienempiä $Foxg1^{cre/cre}$ mutanttialkioilla. Epämuodostumien syytä etsittiin solujen erilaistumisesta ja sitä analysoitiin EDU-värjäyksillä. EDU-värjäykset suoritettiin kallon kehitykselle tärkeissä alkion kehityksen vaiheissa, mutta erilaistumisessa ei havaittu eroja.

Foxg1-mRNA:n ekpressoitumista havaittiin esiaivoissa, mutta ei kehittyvän otsaluun mesenkyymissä. Tämä voi olla merkki siitä, että fenotyyppi voi johtua poikkeavuuksista esiaivojen – ja kallon mesenkyymin kudosinteraktiossa tai jo aiemman vaiheen poikkeavuudesta hermostopienan soluissa.

$Foxg1^{cre/cre}$ mutantien ja $Foxg1^{+/+}$ villityyppien välillä nähtiin poikkeavuuksia HNK1-hermostopienamarkkerin jakaumassa alkioissa iältään 9.5-12.5 päivää. Erot saattavat omalta osaltaan selittää kallon ja kasvojen alueen fenotyyppiä $Foxg1^{cre/cre}$ hiirillä. Hermostopienan vaelluksessa saattaa olla eroa $Foxg1^{cre/cre}$ ja villityypin $Foxg1^{+/+}$ välillä.

Avainsanat – Nyckelord – Keywords

FoxG1, BF1, neural crest cells, mus musculus

Säilytyspaikka – Förvaringställe – Where deposited

1 Abstract

Forkhead box G1 protein (FOXG1) is a transcription repressor that participates in multiple signaling pathways. FOXG1 formerly known as Brain factor 1 (BF1) is known for its effects on neurogenesis. FOXG1 is also expressed in the neural crest cells (NCC's), specifically in the facial NCC's that form the craniofacial structures.

The aim of this study was to investigate the role of FOXG1 in the craniofacial bone development in mice and this was conducted by using a mouse strain with the *Foxg1*-gene targeted by Cre-recombinase (Cre), which represents *Foxg1* null allele. Using a combination of mouse genetics, gene expression analysis and skeletal analysis was used and a difference was seen between mutated *Foxg1*$^{cre/cre}$-embryos (MT) and wild type *Foxg1*$^{+/+}$-embryos (WT).

Abnormalities were found in the frontal region of the skull and in the nasal bones of the *Foxg1*$^{cre/cre}$-embryos. The frontal suture was seen to be star shaped and wider in the mutants and the suture persisted after the WT suture narrowed. The frontal bones and the nasal bones were narrower, and the skulls were smaller in the MT – embryos compared to WT littermates. To analyze whether alteration in cell proliferation could account for these malformations, EDU-stainings at key stages of calvarial development were performed, but no abnormalities were found.

Foxg1-mRNA was detected in the forebrain but not in the developing frontal bone mesenchyme. This might indicate that the phenotype is caused due to abnormalities possible in forebrain – calvarial mesenchyme tissue-tissue interactions or earlier due to abnormalities in the NCC's.

Between embryonic ages 9.5-12.5 (E9.5-E12.5), abnormalities in the distribution of the NCC marker HNK1 were noted in the *Foxg1*$^{cre/cre}$ mutant mice compared to *Foxg1*$^{+/+}$ wild type. The differences may go some way to explain the craniofacial phenotype in these mice.

2 Introduction

Forkhead box G1 protein (FOXG1) is a transcription repressor that participates in multiple signaling pathways. Formerly, it was known as Brain factor 1 (BF1) and is known for its effects on neurogenesis. In humans, mutations in the gene coding FOXG1 causes FoxG1-

syndrome (MIM #613454), which is a Rett-syndrome like disorder that effects many different parts of the development of head structures. These include: postnatal microcephaly, severe mental retardation, absent language, dyskinesia and corpus callosum hypogenesis. ([1]) In *mus musculus* the *Foxg1*$^{cre/cre}$ – embryos (*Foxg1* null allele mice, MT) develop to term, but don't survive after birth. During brain development FOXG1 is essential for telencephalic specialization. ([2])

FOXG1 also regulates the development of the olfactory system. There are no recognizable olfactory structures in *Foxg1* null allele mice. ([3]) This is in alignment that FOXG1 affects the development of neurons and forebrain structures.

Facial Neural Crest Cells (FNCC's) emerge from the posterior diencephalon down to rhombomere two during neurulation. The FNCC's migrate towards the facial area and form osteoblasts, chondrocytes, odontoblasts, myocytes, melanocytes, cranial parasympathetic neurons and their support cells, glia- and some sensory neurons. FNCC's contribute to the craniofacial skeleton and the dentine and the cementine of teeth. They also form the musculoconnective cells to line the endothelium of the blood cells of the face and forebrain. During the migration FNCC's also shield and protect the brain against the effects of the bone morphogenic proteins (BMP's).([3-7]) FOXG1 is expressed in the forebrain, and the neural crest cells (NCC's). FOXG1 is expressed in the prosencephalic neuroepithelium and is involved in the segregation of telencephalon from the diencephalon. In mice, FOXG1 first appear in the five-somite stage at the neural plate. By the embryonic age E10.5 the telencephalic region is cover with FOXG1, excluding the caudal telencephalon. ([4,8,9,10])

FOXG1 controls forebrain development by regulatory WNT86. FOXG1 is regulated by FGF signaling and FOXG1 activity is regulated by *Gli* transcription factors ([11]). Abnormal FGF, WNT and HH (Gli3) signaling are known to recall in calvarial bone phenotypes inducing craniosynostosis and widened sutures. ([12,13,14])

The aim of this study is to investigate the role of FOXG1 in the development of the craniofacial skeleton. It was found that *Foxg1* null allele mutant mice display a calvarial phenotype, the frontal suture is wider than in wild type mice and star shaped in pattern. The mutants also have a smaller skull and narrower nasal bones. The NCC marker HNK1 was found to be altered in *Foxg1* mutants, suggesting that abnormalities in NCC may contribute

to the phenotype observed. It was also shown that FOXG1 is not expressed in the developing frontal bone itself, but in the adjacent underlying forebrain.

3 Materials and methods

3.1 Ethical issues

All experiments were approved by the University of Helsinki (KEK14-05) and The Southern Finland Council of Animal Welfare and Ethics (ESAVI/10370/04.10.07/2014).

3.2 Mice

The study was conducted using a mouse strain with the FoxG1-gene targeted by Cre-recombinase (Cre). Mice were maintained as heterozygotes as homozygotes are not viable postnatally. ([2,15]).

3.3 Genotyping

All embryos were genotyped, from liver samples or tail samples using primers for FoxG1 and Cre. DNA was extracted by a fast extraction – method: incubated in 95°C for one hour in 75µl of alkaline lysis reagent (NaOH 0.5g; Na_2-EDTA $2H_20$ 0.03722g; pH~12), after incubation samples were cooled on ice and 75µl of neutralizing reagent (Tris-HCl 3.152g, pH~5) was added and samples were ready for PCR. PCR was conducted with Thermo Fischers Dynazyme buffer and Dynazyme II polymerase. Primers used: FoxG1-F GAACGGCAAGTACGAGAAGC, FoxG1-R TCACGAAGCACTTGTTGAGG product 197 base pairs; Cre-F AATCTCCCACCGTCAGTACG, Cre-R CGTTTTCTGAGCATACCTGGA product 472 base pairs.

3.4 Immunohistochemical staining of Neural crest cells

In embryonic development the bones of the face, the frontal bones and the anterior part of the skull base are formed by the neural crest cells (NCC) ([4,16]). In this study, NCC's were mapped at embryonic (E) days 10.5 and 12.5 using a beta-1,3-glucuronyltransferase 1 (HNK1)-antibody. HNK1- is an early NCC-marker ([16]). $FoxG1^{cre/cre}$-embryos (MT) were compared to $FoxG1^{+/+}$-embryos (WT) to see if there is a difference in the migration and mapping of the NCC's. The whole embryos were fixed in 4%-paraformaldehyde (PFA)-solution overnight and dehydrated in rising methanol series. The embryos were rehydrated in a rising methanol series, washed with Tris Buffered Saline-Tween (0.1%) – solution

(TBST). After this the endogenous peroxidase activity was diminished by incubating the embryos in 6% hydrogen peroxide solution in TBST for 30 minutes in room temperature (RT) and washed in TBST after. Then the embryos were treated with proteinase K 10g/ml solution in TBST for 20 minutes in RT and washed with TBST. Embryos were then blocked in a solution containing 5% bovine serum albumin (BSA) and 3 % fetal bovine serum in TBST overnight at 6°C. Then the embryos were incubated with the primary antibody (HNK1) 1:1000 in TBST with 3% BSA overnight at 6°C and washed with TBST. After this the embryos were incubated over night at 6°C with the secondary antibody 1:1000 in TBST with 3% BSA, which was horseradish peroxidase – fluorescent conjugated (provided in the EnzMet™ -kit). Then the embryos were treated according to EnzMet™ HRP Detection Kit for IHC / ISH, and imaged.

3.5 EDU proliferation assay

The proliferation of cells was detected by an 5-ethynyl-2'-deoxyuridine (EDU)-assay. Time-mated females were injected intraperitoneally with EDU 100µl, 10mg/ml, 5µl/g. two hours before harvesting embryos. Embryos were dissected, keeping only the heads, fixed overnight in a 4%-paraformaldehyde (PFA)-solution, dehydrated in a rising ethanol series and xylene and embedded in paraffin. After embedding the tissues were sectioned at 7µm intervals. Sections were deparaffinated in xylene and rehydrated in a rising ethanol series 100%, 95% and 80 % for 10 minutes each. Sections were rinsed in deionized water and washed with 2mg/ml glycine-solution. After washing sections were permeabilized with 0,5% Triton X-100 in Dulbecco's phosphate-buffered saline (PBS) (pH 7.3) for 20 minutes and washed with PBS twice for 10 minutes. Sections were incubated in Click-iT ™ reaction- cocktail in darkness for 30 minutes and rinsed twice with PBS for 10 minutes. DNA-staining was also conducted with Hoechst 33342 5µl/ml in PBS - solution also incubated for 30 minutes in darkness. After incubation sections were washed with PBS twice for three minutes and mounted with Immumount™. EDU-proliferation assays were made to embryos aged E13.5 and E15.5 to see if there is a difference in the proliferation of cells in MT-embryos compared to the cells in WT-embryos. The cells were calculated from 20x magnification on an area of $1cm^2$. The ratio between cells that are dividing, and all cells were calculated.

3.6 *In situ*-hybridisations

Probe template was generated from a plasmid containing the FoxG1 m-RNA, by PCR from an MGC cDNA (accession BC046958) using primers, lower case letter indicates the phage promoter: FoxG1F AGT TAC AAC GGG ACC ACG TC and FoxG1R-T3 aattaaccctcactaaagg CCCTGATTTTGATGTGTGA. cDNA-clone was processed to antisense(AS) and sense (S) – coding templates with primers: AS FoxG1-F-T7 TAATACGACTCACTATAGGGAGTTACA ACGGGACCA CGTC, FoxG1-T7R GAAGACCCCTGATTTTGATGTGTG; S FoxG1-F1 AGTTACAACGGGACCACGTC, FoxG1-T3 R AATTAACCCTCACTAAAGGCCCCTGATTTTGATGTGTGA. The transcription template was generated from the cDNA clone using T3 and T7 primers and labelled with digoxigenin (DIG). ([17])

DIG – labelled FoxG1- mRNA-probe was made and a paraffin section *in situ*-hybridisation was conducted to embryo heads aged E12.5 WT-embryos. It was shown that FoxG1 is expressed only in the brain, not in the skull as shown previously. ([2,15,16]) Whole mount in situ-hybridisations were made WT-embryos aged E9.5, E10.5 and E12.5 to map out the expression patterns of FoxG1, using the same mRNA-probes. ([17])

3.7 Skeletal preparations

Skeletal preparations were made of embryos aged E15.5, E18.5 and new born pups (NB), to show the phenotype. The skeletons were stained with Alcian blue, which stains the cartilage and Alizarin red, which stains the mineral/bone. Embryos were fixed overnight in 95% ethanol in 20ml scintillation vials, after removing the skin. After fixation the embryos were incubated in one volume glacial acetic acid and four volumes 95% ethanol and 7,5 mg/50ml Alcian blue for 48 hours. After incubation embryos were rinsed in 95% ethanol for one hour and incubated in 2% potassium hydroxide (KOH)-solution (E15.5 for one hour, E18.5 and NB for 6 hours). 2%KOH-solution was replaced by 1%KOH solution with 75µg/ml Alizarin red and incubated overnight. After these incubations embryos were cleared for a week in 20% glycerol-1%KOH – solution. For storage embryos were transferred to 50%glyserol-50%ethanol – solution.

3.8 Micro computed tomography (µCT)

Micro CT is three-dimensional x-ray imaging. NB embryo heads were fixed in an ethanol series and embedded in an agarose gel. The system used in the scanning was Bruker's scanner: SkyScan1272 and the software version was 1.1.1. The imaging parameters were: Camera Pixel Size (µm) = 9.0, Camera X/Y Ratio: 1.0118, Source Voltage (kV) = 60, Source Current (µA)= 166, Reference Intensity=57000, Exposure (ms)=512, Rotation Step (degrees)=0.300.

3.9 Statistical analysis

P-values were calculated with a paired t-test and $p<0.05$ was considered statistically significant and $p<0.001$ considered to be statistically highly significant.

4 Results

4.1 *Foxg1*$^{cre/cre}$ mutant mice exhibit nasal and frontal bone abnormalities

In the skeletal preparations was found that there is a skull phenotype in the *Foxg1*$^{cre/cre}$ mutant embryos (MT) compared to wild type (WT) litter mates. (Figure 1.) The rest of the skeleton was unaffected.

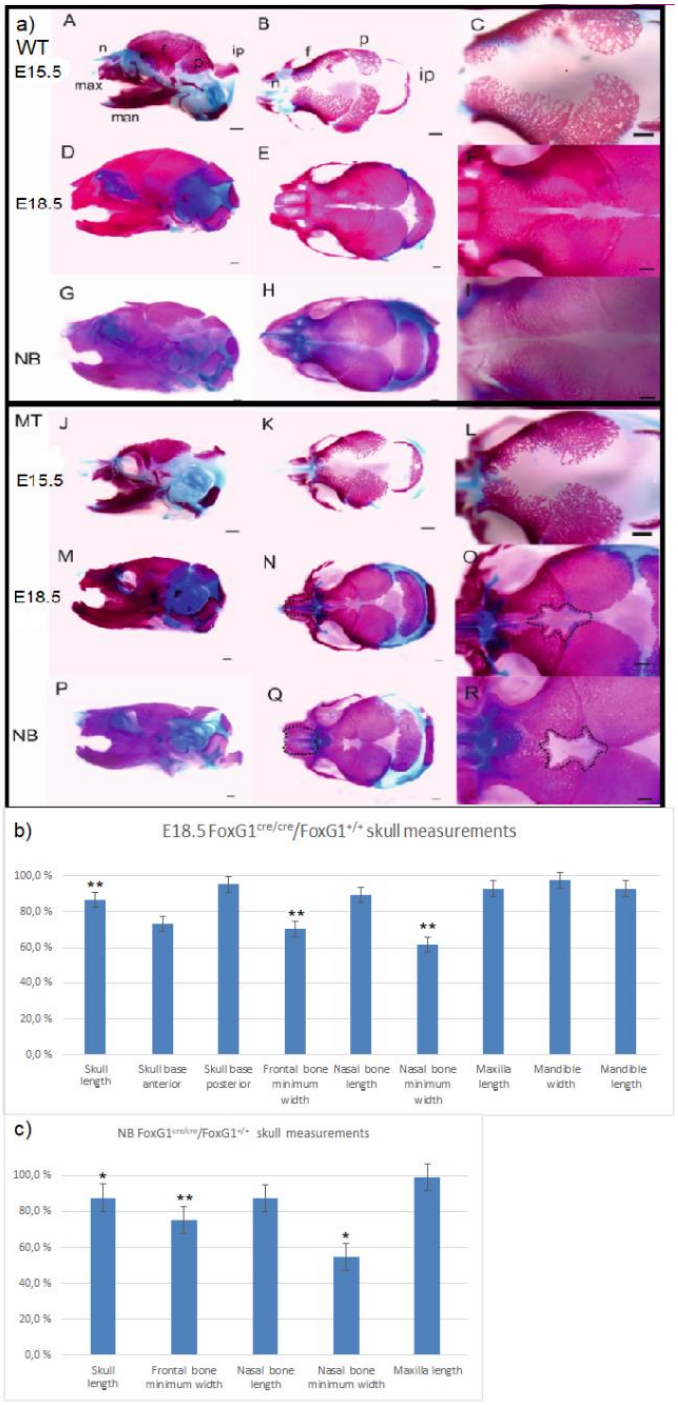

Figure 1. FoxG1$^{cre/cre}$ mice exhibit skeletal defects in the nasal and frontal bones. Alcian blue/Alizarin red – staining of Foxg1 $^{+/+}$ - embryos (a) A-I) Foxg1 $^{cre/cre}$ - embryos (a) J-R). A, D, F, J, M and P is showing the side image of the stained skulls, in MT-embryos the maxilla appears retruded compared to the mandible, as in the WT-embryos the maxilla and mandible are well balanced. B, E, H, K, N and Q is showing the calvaria, dotted black lines in N and

Q indicate the unusual shape of the nasal bones, which are narrower than in the WT-embryos E and H. C, F, I, L, O and R is showing the calvaria, dotted black lines in O and R indicate the star shape of the suture, in F and I the WT-embryos suture doesn't have the star shape. b) and c) illustrates the measurements shown as a percentage of MT/WT. Single asterisk indicate the statistical significance of p<0.05, and two asterisks indicates the statistical significance of p<0.001, error bars indicate standard error. The scale bar in all the pictures is 0.5mm. Magnifications in A, D, F, J, M, Q, B, E, H, K, N and Q is 1.25; in C and L 3.2; in F, I, O and R 2.5. f=frontal bone, ip=inter parietal bone, n=nasal bone, man=mandible, max=maxilla, p=parietal bone.

A series of measurements was conducted from the skeletal preparation images. It was found that the skull was slightly shorter in the MT-embryos compared to the WT-embryos. Also, the anterior part of the skull base, nasal bone width and the frontal bone minimum width were smaller in the MT-embryos. (Figure 1 a) and b)) A p-value of 0.05 was considered statistically significant and it was calculated with a paired t-test. Measurements are seen in figure 1 b) and c) as a percentage of MT compared to WT. Whole skull is measured from the anterior tip of the nasal bone to the posterior tip of occipital bone (occiput) and the p-values: E18.5 4.8*10^-10, NB 0.002. Skull base anterior: E18.5 p-value 0.2 and Skull base posterior: E18.5 p-value 0.2. Minimum width of frontal bones: E18.5 p-value 1.7*10^-09, NB p-value 0.001. Nasal bone length: E18.5 p-value 0.2, NB p-value 0.08. Minimum width of nasal bones: E18.5 p-value 1.1*10^-06, NB p-value 0.005. Mandible width: E18.5 p-value 0.8 and mandible length: E18.5 p-value 0.06. Maxilla length: E18.5 p-value 0.07, NB p-value 0.9. Statistically significant difference between mutant, and wild type at ages E18.5 and NB in whole skull size, frontal bone width and nasal bone. The number of embryos in the age E18.5 is 10 MT and seven WT. The number of embryos in NB's was four MTs and three WTs.

Differences seen in alizarian red/alcian blue stainings was also seen in the µCT-images (Figure 2) The nasal bones, skull base and the frontal bones are affected by the loss of FOXG1. In addition, another abnormality in the frontal bones was found in the µCT-images. The frontal bones in the MT-embryos are two layered (Figure 2 J).

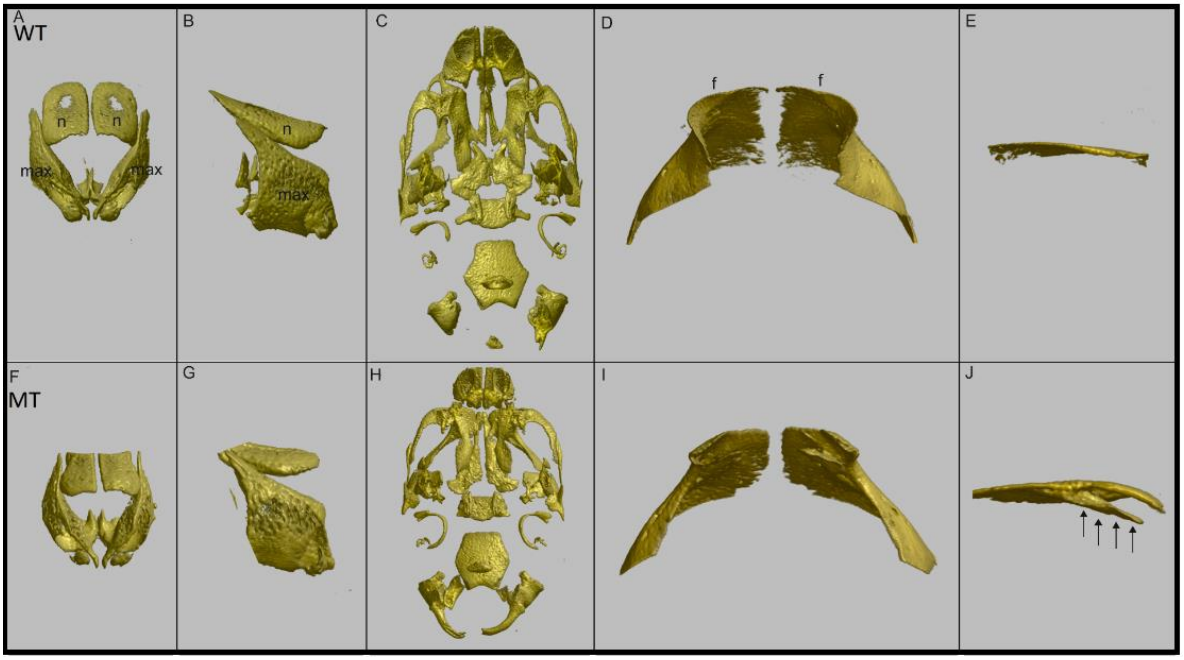

Figure 2. Nasal bone and frontal bone phenotype of Foxg1$^{cre/cre}$-embryos. µCT – images of NB-embryos of MT Foxg1 $^{cre/cre}$ - embryos (F-J) and WT Foxg1 $^{+/+}$ - embryos (A-E). A, B, F and G are showing the nasal bones. C and H are showing the skull bases. D. E, I and J are showing the frontal bones. The arrows in J are illustrating the frontal bone phenotype: in the MT the bone is in two layers as in the WT (E) the bone is one layered. f=frontal bone, n=nasal bone, max=maxilla.

4.2 *Foxg1* mRNA is not expressed in the developing frontal bone

Foxg1 mRNA was detected by *in situ*-hybridisation in the developing brain and limbs (Figure 3). Despite *Foxg1*$^{cre/cre}$ mutation the mice exhibit a frontal bone. Phenotype *Foxg1* was not detected in the frontal bone primordia at E12.5. FOXG1 was however expressed in the other cells of the forebrain. A location adjacent to calvarial mesenchyme into which the frontal bone will develop. This directs to the hypothesis that possible forebrain calvarial mesenchyme tissue-tissue interactions may regulate frontal bone development.

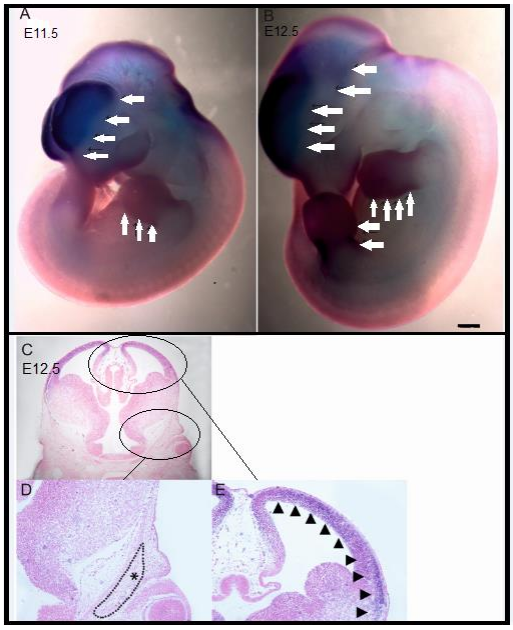

Figure 3. FoxG1 is expressed in the forebrain and limbs of NMRI-WT-mice. A and B is showing whole mount in situ-hybridisation of FoxG1 antisense mRNA-probe in NMRI-WT mice E11.5 and E12.5. Scale bar 0.5mm. Arrows indicate the expression present in the forebrain area, and in the fore and hind limbs. C-E Expression pattern of FoxG1 on paraffin section at E12.5 Foxg1 $^{+/+}$ *- embryo with 4x magnification in C), 10x magnification in D) and E). C) Shows the frontal section of the embryo upper ellipse is showing the magnification point of E) and the lower ellipse is showing the magnification point of D). D) The area with the asterisk is showing the frontal bone, which doesn't have any expression of FoxG1. E) The arrowheads are marking the expression in the brain, but the expression is not seen in the surface structures.*

A whole mount *in situ*-hybridisation was also conducted and it was found that the expression pattern was as shown before: in the limbs and brain. (Figure 4.)

4.3 Increased expression of neural crest cell marker HNK1 in *Foxg1* $^{cre/cre}$ mutant mice

FOXG1 is known to be expressed in cranial NCC's. To test whether NCC distribution is altered in *Foxg1*$^{cre/cre}$ mutants, whole mount samples from *Foxg1* $^{cre/cre}$ and WT littermates were stained by immunohistochemistry for the NCC marker HNK1 at key stages of NCC migration (E9.5-E12.5). In the HNK1- antibody staining a difference was seen in the expressions. It was found that in the earlier stages (E9.5) of the migration of the NCC's there

is slightly more expression seen in the MT-embryos. At E10.5 there seems to be more expression in the WT and then in the E12.5 embryos there is an obvious difference in the MT-embryos compared to the WT-embryos. The E12.5 expression difference is significantly seen in the facial area.

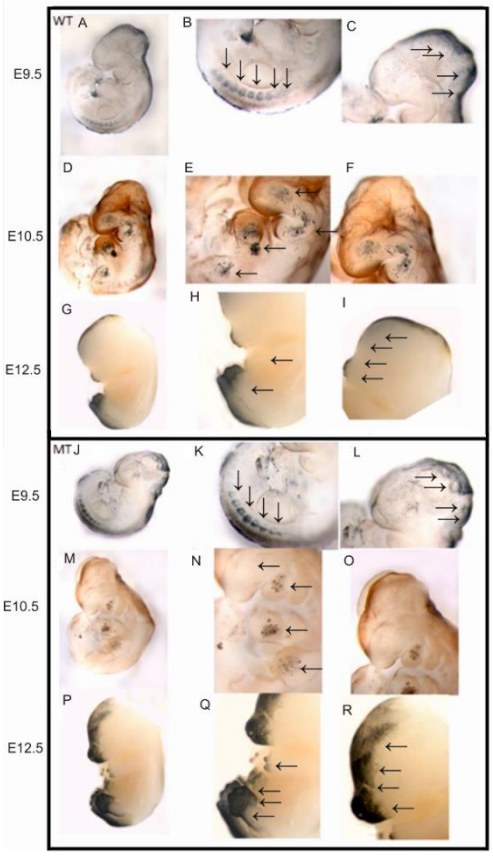

Figure 4. The expression of HNK1, a NCC-marker, in Foxg1 $^{cre/cre}$ (MT) - and Foxg1 $^{+/+}$ (WT) -embryos. All the pictures illustrate whole mount HNK1-Immunohistochemical staining. The arrows in B, C mark the difference in the WT- embryos compared to K, L MT-embryos at E9.5. It is seen that, the expression in the MT (K) is only in eight vertebrae as in the WT (B) the expression is in 10. There is a difference also in the expression in the back of the developing head. The arrows in C and L indicate the difference, in the MT (L) the pattern is wider and, in the WT (C), the pattern is more stricted and darker. The arrows in E and N show the difference at E 10.5, there is less expression in the MT (N) than in the WT (E). Then in the E12.5 the expression in the MT (Q and R) is significantly wider and stronger than in the WT (H and I).

4.4 Proliferation in *Foxg1*$^{cre/cre}$ mutant mice

In the EDU-proliferation assay there were no significant differences found between MT- and WT-embryos. The cells were calculated with a fixed area and the area was chosen to be the caudal part of the developing frontal bone (Figure 5).

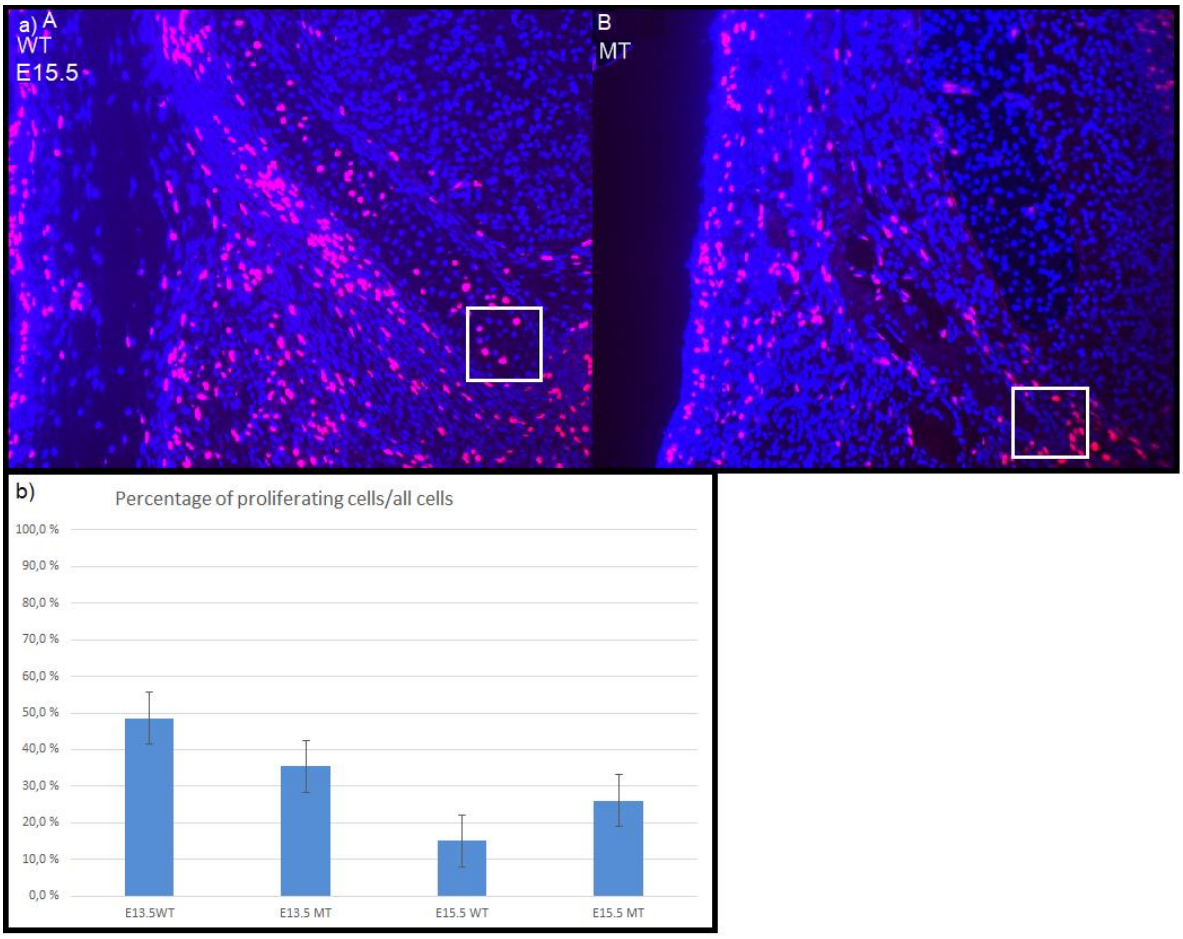

Figure 5. There seems to be no difference in the proliferation of cells in Foxg1 $^{cre/cre}$ *(MT) -embryos compared to the Foxg1* $^{+/+}$ *(WT) -embryos. a) Is showing a frontal sections of WT (A) and MT (B). The rectangles indicate the placement of calculated cells. b) shows the ratio between proliferating cells divided with the amount of all cells at ages E13.5 and E15.5, error bars indicate standard error.*

5 Discussion

5.1 Introduction

Craniofacial development is a complex process which involves many tissue interactions and signaling pathways. The consequences of disruption of these processes can cause many different craniofacial abnormalities. ([18])

In this study we report that frontal bone development is affected *Foxg1*$^{cre/cre}$ mutant mice. The tissue affected arises from the neural crest cells (NCC's). ([5])

5.2 Key findings

In late embryonic stages the frontal bones approximate each other, the frontal suture narrows, and the anterior fontanelle reduces in size, however in *Foxg1* null allele mutants there is an enlargement of this suture and frontanelle. There also was an abnormal shape in the in frontal suture of the *Foxg1* $^{cre/cre}$ (MT) – embryos. It was also revealed that the frontal bones of the MT-embryos were curved/two layered and the frontal bones were narrower than in the *Foxg1* $^{+/+}$ (WT)-littermates. The skull was shorter in the in MT-embryos compared to the in (WT) littermates. The MT-embryos had also narrower nasal bones.

It was seen in the *in situ*-hybridisations that FOXG1 is expressed in the forebrain, but not in the developing calvaria, as was shown before. ([8,9,10]) The expression of FOXG1 was also seen in the fore and hind limbs of the mice. This expression pattern leads to the hypothesis, that forebrain – calvarial mesenchyme tissue-tissue interactions may regulate the developing frontal bone. Interactions between the developing brain and calvaria have been suggested previously to regulate calvarial osteogenesis. ([19]) Signals from the dura mater are known to regulate calvarial suture pattern. ([20,21])

The affected area is in the region of NCC's. In the NCC marker HNK1- antibody stainings a difference was seen in the expressions between MT and WT-littermates. It was found that in the earlier stages (E9.5) of the migration of the NCC's there is slightly more expression seen in the MT-embryos. At E10.5 there seems to be more expression in the WT. At the age of E12.5, the expression has an obvious difference in the MT-embryos compared to the WT-embryos. The area that has the most expression is the facial area, that gives rise to the structures that have abnormalities in the MT-embryos. It has been shown that alterations in

calvarial cell proliferation can result in delayed osteogenesis and enlarged sutures ([15]). However, *Foxg1* $^{cre/cre}$ mice did not show proliferation abnormalities in the areas assayed, which could count for the abnormalities seen. Therefore, the hypothesis that the migration of the NCC's is affected by the lack of FOXG1, would need more experimentation to be verified.

Results described here have allowed to be speculated that the frontal bone phenotype exhibited by *Foxg1*$^{cre/cre}$ mice may be the result of alteration in NCC migration or further and/or for disruption of tissue-tissue interaction between the developing brain and the calvarial mesenchyme. Within the presented framework it is limited to these two hypotheses.

5.3 Significance

In humans, mutations in the gene coding FOXG1 cause Foxg1-syndrome, which is a Rett-syndrome like disorder that effects many different parts of the development of head structures. These include: postnatal microcephaly, severe mental retardation, absent language, dyskinesia and corpus callosum hypogenesis. ([1]) Although relevantly few *FOXG1*-syndrome patients have been documented more are being described and knowledge of the craniofacial phenotype in mice mutants: abnormal shape and enlargement of the frontal suture and frontanelle, may be useful to patients and diagnosing clinicians. ([22])

5.4 Conclusions

A difference between the MT and WT embryos was found in the skull. The frontal suture developed into an abnormal shape, and the frontal bones formed into two-layered/curved and were narrower. Also, the width of the nasal bones of the MT-embryos were significantly narrower than in the WT-embryos. The proliferation assay did not show a statistically relevant difference in the proliferation of the cells which leads to the assumption that abnormalities observed in NCC's may explain, at least in part, the phenotype described. Verification of this hypothesis and integration with signaling pathways in which FOXG1 is known to act, including WNT-signaling, will need further investigation. ([11,17])

The mouse's frontal suture equivalent in humans is the metopic suture and it is normally fused in the first year after birth. Knowledge of the phenotype in *Foxg1*$^{cre/cre}$ mice will be of interest, not only to investigators studying craniofacial development, but also to patients with *FOXG1*-mutations and their attending clinicians. If similar defects seen in *Foxg1*$^{cre/cre}$ mice

were detected in patients, steps may be taken to protect the brain underlying an abnormally large interfrontal/metopic suture. ([23,24])

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8 References

1. Kortum F, Das S, Flindt M, et al. The core FOXG1 syndrome phenotype consists of postnatal microcephaly, severe mental retardation, absent language, dyskinesia, and corpus callosum hypogenesis. *J Med Genet*. 2011;48(6):396-406. doi: 10.1136/jmg.2010.087528 [doi].

2. Targeting of cre to the Foxg1 (BF-1) locus mediates loxP recombination in the telencephalon and other developing head structures. http://www.sciencedirect.com/science/article/pii/S001216060099732X. Accessed 6/4/2015, 2015.

3. Duggan,C.D., DeMaria,S., Baudhuin,A., Stafford,D., Ngai,J. Foxg1 is required for development of the vertebrate olfactory system. J.Neurosci., 2008, 28, 20, 5229-5239, United States.

4. Wang Y, Song L, Zhou CJ. The canonical wnt/beta-catenin signaling pathway regulates fgf signaling for early facial development. *Dev Biol*. 2011;349(2):250-260. doi: 10.1016/j.ydbio.2010.11.004 [doi].

5. Aguiar DP, Sghari S, Creuzet S. The facial neural crest controls fore- and midbrain patterning by regulating Foxg1 expression through Smad1 activity. *Development*. 2014;141(12):2494-2505. doi: 10.1242/dev.101790 [doi].

6. Anderson RM, Lawrence AR, Stottmann RW, Bachiller D, Klingensmith J. Chordin and noggin promote organizing centers of forebrain development in the mouse. *Development*. 2002;129(21):4975-4987.

7. Creuzet,S.E. Regulation of pre-otic brain development by the cephalic neural crest. Proc.Natl.Acad.Sci.U.S.A., 2009, 106, 37, 15774-15779, United States.

8. Manuel,M., Martynoga,B., Yu,T., West,J.D., Mason,J.O., Price,D.J. The transcription factor Foxg1 regulates the competence of telencephalic cells to adopt subpallial fates in mice. Development, 2010, 137, 3, 487-497, England.

9. Hatini,V., Tao,W., Lai,E. Expression of winged helix genes, BF-1 and BF-2, define adjacent domains within the developing forebrain and retina. J.Neurobiol., 1994, 25, 10, 1293-1309, United States.

10. Tao,W., Lai,E. Telencephalon-restricted expression of BF-1, a new member of the HNF-3/fork head gene family, in the developing rat brain. Neuron, 1992, 8, 5, 957-966, United States.

11. Danesin C, Houart C. A fox stops the wnt: Implications for forebrain development and diseases. *Curr Opin Genet Dev*. 2012;22(4):323-330. doi: 10.1016/j.gde.2012.05.001 [doi].

12. Twigg SR, Wilkie AO. A Genetic-Pathophysiological Framework for Craniosynostosis. Am J Hum Genet. 2015 Sep 3;97(3):359-77. doi: 10.1016/j.ajhg.2015.07.006.

13. Twigg SR, Wilkie AO. New insights into craniofacial malformations. Hum Mol Genet. 2015 Oct 15;24(R1):R50-9. doi: 10.1093/hmg/ddv228. Epub 2015 Jun 17.

14. Rice DP, Connor EC, Veltmaat JM, Lana-Elola E, Veistinen L, Tanimoto Y, Bellusci S, Rice R. Gli3Xt-J/Xt-J mice exhibit lambdoid suture craniosynostosis which results from altered osteoprogenitor proliferation and differentiation. Hum Mol Genet. 2010 Sep 1;19(17):3457-67. doi: 10.1093/hmg/ddq258. Epub 2010 Jun 22.

15. Eagleson KL, Schlueter McFadyen-Ketchum LJ, Ahrens ET, et al. Disruption of Foxg1 expression by knock-in of cre recombinase: Effects on the development of the mouse telencephalon. *Neuroscience*. 2007;148(2):385-399. doi: S0306-4522(07)00740-3 [pii].

16. Erin Betters, Barbara Murdoch, Alan W. Leung, Martín I. García-Castro. Human neural crest cells and stem cell-based models - neural crest cells - chapter 18. http://www.sciencedirect.com/science/article/pii/B9780124017306000193. Accessed 7/22/2014, 2014.

17. Wei Q, Condie BG. A focused in situ hybridization screen identifies candidate transcriptional regulators of thymic epithelial cell development and function. *PLoS One*. 2011;6(11):e26795. doi: 10.1371/journal.pone.0026795 [doi].

18. Rice, D.P., Rice,R., Thesleff,I. Molecular mechanisms in calvarial bone and suture development, and their relation to craniosynostosis. Eur.J.Orthod., 2003, 25, 2, 139-148, England.

19. Schowing J. Inductive influence of the embryonic brain on the development of the skull in the chicken. I. Influence of excision of the anterior nervous area on cranial development. J Embryol Exp Morphol. 1968 Feb;19(1):9-22.

20. Opperman LA, Sweeney TM, Redmon J, Persing JA, Ogle RC. Tissue interactions with underlying dura mater inhibit osseous obliteration of developing cranial sutures. Dev Dyn. 1993 Dec;198(4):312-22.

21. Bradley JP, Levine JP, Roth DA, McCarthy JG, Longaker MT. Studies in cranial suture biology: IV. Temporal sequence of posterior frontal cranial suture fusion in the mouse. Plast Reconstr Surg. 1996 Nov;98(6):1039-45.

22. Mencarelli,M.A., Spanhol-Rosseto,A., Artuso,R., et al. Novel FOXG1 mutations associated with the congenital variant of Rett syndrome SourceJ.Med.Genet., 2010, 47, 1, 49-53, England.

23. Caporali, C., Signorini,S., De Giorgis,V., et al. Early-onset movement disorder as diagnostic marker in genetic syndromes: Three cases of FOXG1-related syndrome. Eur.J.Paediatr.Neurol., 2018, 22, 2, 336-339, European Paediatric Neurology Society. Published by Elsevier Ltd, England.

24. Allou, L., Lambert,L., Amsallem,D., et al. 14q12 and severe Rett-like phenotypes: new clinical insights and physical mapping of FOXG1-regulatory elements. Eur.J.Hum.Genet., 2012, 20, 12, 1216-1223, England.